Please type a plus sign (+) inside this box →

PTO/SB/05 (4/98)
Approved for use through 09/30/2000. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	11A231.119
First Inventor or Application Identifier	Reid et al.
Title	Proliferation of Hepatocyte...
Express Mail Label No.	

JC759 U.S. PTO 03/24/00

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231

1. [X] * Fee Transmittal Form *(e.g., PTO/SB/17)* *(Submit an original and a duplicate for fee processing)*
2. [X] Specification [*Total Pages* 21]
   (preferred arrangement set forth below)
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings *(if filed)*
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [X] Drawing(s) *(35 U.S.C. 113)* [*Total Sheets* 0]
4. Oath or Declaration [*Total Pages* 4]
   a. [ ] Newly executed (original or copy)
   b. [X] Copy from a prior application (37 C.F.R. § 1.63(d)) *(for continuation/divisional with Box 16 completed)*
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

5. [ ] Microfiche Computer Program *(Appendix)*
6. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
   a. [ ] Computer Readable Copy
   b. [ ] Paper Copy (identical to computer copy)
   c. [ ] Statement verifying identity of above copies

JC135 U.S. PTO 09/534487 03/24/00

ACCOMPANYING APPLICATION PARTS

7. [X] Assignment Papers (cover sheet & document(s))
8. [ ] 37 C.F.R.§3.73(b) Statement *(when there is an assignee)* [ ] Power of Attorney
9. [ ] English Translation Document *(if applicable)*
10. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
11. [X] Preliminary Amendment
12. [X] Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)*
13. [ ] * Small Entity Statement(s) *(PTO/SB/09-12)* [X] Statement filed in prior application, Status still proper and desired
14. [ ] Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
15. [ ] Other: .....

16. **If a CONTINUING APPLICATION,** *check appropriate box, and supply the requisite information below and in a preliminary amendment:*

[X] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application No: 09/115,920

Prior application information: Examiner E. Kemmerer Group / Art Unit: 1646

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

17. CORRESPONDENCE ADDRESS

[X] *Customer Number or Bar Code Label* *(Insert Customer No. or Attach bar code label here)* 21269 or [ ] *Correspondence address below*

Name	PATENT TRADEMARK OFFICE				
Address					
City		*State*		*Zip Code*	
Country		*Telephone*		*Fax*	

Name (Print/Type)	Gilberto M. Villacorta, Ph.D	*Registration No. (Attorney/Agent)*	34,038
Signature	Gilberto M. Villacorta	*Date*	03-24-00

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

Applicant or Patentee: Reid, et al.
Serial or Patent No.: Unknown
Filed or Issued: November 18, 1996
For: PROLIFERATION OF HEPATOCYTE PRECURSORS

Docket No: 96700/426

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[X] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN Renaissance Cell Technologies, Inc.
ADDRESS OF CONCERN 3621 Sweeten Creek Road
Chapel Hill, NC 27514

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled PROLIFERATION OF HEPATOCYTE PRECURSORS by inventor(s) Reid, et al. described in

[ ] the specification filed herewith
[X] application serial no. Unknown, filed November 18, 1996
[ ] patent no. _____, issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME _____
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Dr. Lola M. Reid
TITLE OF PERSON OTHER THAN OWNER President
ADDRESS OF PERSON SIGNING 3621 Sweeten Creek Road
Chapel Hill, NC 27514
SIGNATURE X Lola M Reid DATE November 18, 1996

Docket No. 114231.119

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	
	:	
REID et al.	:	
	:	
Serial No.	:	Group Art Unit:
	:	
Filed:	:	Examiner:
	:	

For: PROLIFERATION OF HEPATOCYTE PRECURSORS

PRELIMINARY AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

Preliminary to examination of the above-referenced application, please amend the application as follows:

IN THE SPECIFICATION

Please amend the specification as follows:

Page 1, after the title please insert

-- Related Application

This application is a continuation of US Application No. 09/115,920 filed July 15, 1998 which is a continuation of US Application No. 08/751,546 filed November 18, 1996, now Patent No. 5,789,246, which is a divisional of US Application No. 08/165,696 filed June 25, 1996, now Patent No. 5,576,207, which is a continuation of US Application No. 07/741,128 filed August 7, 1991, now abandoned, each of which is herein incorporated by reference.--

Docket No. 114231.119

AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for this Amendment, or credit any overpayment to deposit account no. 50-0436.

In the event that an extension of time is required, or which may be required in addition to that requested in a petition for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to deposit account no. 50-0436.

Respectfully Submitted,

PEPPER HAMILTON LP

Gilberto M. Villacorta, Ph.D.
Registration No. 34,038

600 Nineteenth Street, NW
Washington, DC 20005
(202) 220-1200 GMV:cgm
Date:
Facsimile: 202-220-1201
DC: #144826 v1 (33QY01!.WPD)

PROLIFERATION OF HEPATOCYTE PRECURSORS

This invention relates to the expansion, or proliferation of cells and in particular cells whose progeny may differentiate into mature hepatocytes. More particularly, this invention relates to the enrichment of, and to the expansion or proliferation of such cells in the presence of stromal cells, an extracellular matrix, and/or growth factors. In another aspect, this invention relates to genetically engineered cells which are capable of differentiating into hepatocytes.

In accordance with an aspect of the present invention, there is provided a composition which comprises an animal cell population. The cell population contains immature cells (i) at least a portion of said cells or a portion of the progeny of said cells is capable of differentiating into hepatocytes and (ii) which are characterized by expression of alpha-fetoprotein or lack of essential expression of alpha-fetoprotein and albumin, and at least a portion of said cells or of the progeny of said cells is capable of differentating into cells which express albumin. In general, the differentiated cells which express albumin have morphological and physiological characteristics of mature hepatocytes. The cell population has been cultured under conditions which result in expansion of the immature cells. Such cells are sometimes hereinafter referred to as "hepatocyte precursors".

The hepatocyte precursors may be derived from any animal, preferably from mammals. Mammals from which the hepatocyte precursors may be derived include, but are not limited to, humans, rodents (e.g., rats, mice, hamsters), rabbits, bovines, horses, pigs, and sheep. Preferably, the hepatocyte precursors are derived from humans.

Although the hepatocyte precursors are preferably obtained from liver tissue, such cells may be obtained from other sources, such as, but not limited to, the pancreas, gut, lung, and bone marrow.

In general, such hepatocyte precursors may be obtained from an excised section of liver. The excised section of liver may then be dissociated by standard procedures into single dissociated cells. Such procedures include enzymatic dissociation and mechanical dissociation. Enzymatic dissociation may be carried out in the presence of protease(s), such as collagenase(s) and/or nuclease(s), such as DNase. In some instances, pronase(s) may also be used. Such pronase(s) also contribute to the enrichment of hepatocyte precursors. An example of enzymatic dissociation of liver cells is described in Pretlow, et al., eds., Cell Separation: Methods and Selected Applications, pgs. 45-77, Academic Press, New York (1987). The cells are then subjected to an enrichment procedure to eliminate mature liver cells from the cell population. Various procedures exist for enrichment. Such procedures include, but are not limited to, enzymatic digestion with pronase, DNase, and collagenase; centrifugal elutriation for cells which are smaller than mature hepatocytes; and freezing the cells in liquid nitrogen in the presence of 10% glycerol. It is to be understood, however, that the scope of the present invention is not to be limited to cells of a specific size range or a specific morphology.

Alternatively, the immature cells may be enriched by contacting cells from an excised section of liver tissue, or of other tissue, which may contain the hepatocyte precursor cells, with monoclonal antibodies which recognize an epitope of the hepatocyte precursor cells. Such cells may then be separated from the remainder of the cells of the excised tissue by procedures known to those skilled in the art.

One example of an enrichment procedure entails obtaining a liver section, and placing the liver section in an ice-cold saline solution which may contain buffers, glucose, and/or antibiotics.

The liver section is then minced and sequentially digested with a solution containing collagenase, pronase, and deoxyribonuclease, prepared in a saline solution to which CaCl 2 is added. The digestions preferably are done at 37°C in a shaking water bath and for a period of time of about 20 minutes. The partially digested tissue is then strained through a tissue sieve by gravity and the undigested remnants are redigested two times as hereinabove described. The collected cells are then washed with saline solution, counted, and assessed for viability.

The enriched hepatocyte precursor population may then be cultured under conditions which result in the expansion, or proliferation of the hepatocyte precursors. Thus, in accordance with another aspect of the present invention, there is provided a process for expanding, or proliferating immature cells characterized as hereinabove described. The process comprises culturing the immature cells under conditions providing for expansion of the immature cells. Preferably, the process comprises culturing the immature cells in the presence of (i) an extracellular matrix and (ii) liver stromal cells. Preferably, the liver stromal cells are embryonic liver stromal cells or fetal liver stromal cells. In general, stromal cells are mesenchymally-derived cells that in vivo are closely associated with and are in a paracrine relationship with epithelia. Stromal cells also grow readily in culture on tissue culture plastic and in serum-supplemented media. In general, such cells also produce fibrillar collagens.

The term "expanding", as used herein, means that the immature cells, or hepatocyte precursors, are cultured under conditions which result in the growth or proliferation of the immature cells.

Examples of extracellular matrix components include, but are not limited to collagen, such as, for example, collagen Type IV, or the adhesion proteins fibronectin, and laminin. A preferred extracellular matrix component is collagen Type IV. The collagen, when employed, may be used alone or in combination with laminin or fibronectin, or in combination with proteoglycans, or with tissue extracts enriched in extracellular matrix materials.

Preferably, the extracellular matrix component is coated upon a porous solid support. Examples of porous solid supports which may be employed include, but are not limited to porous supports such as Millicell membrane supports, filters, sponges, and hollow fiber systems. Alternatively, the extracellular matrix may be unattached to the porous solid support. Examples of such matrices include floating collagen gels, gel foams, spheres of synthetic materials or - fibers of synthetic materials such as dextran, polystyrene, and agarose.

The hepatocyte precursors are cultured in a suitable basal medium, preferably a serum-free medium. More preferably, the medium has a calcium content of less than 0.4mM. Examples of such media include, but are not limited to, Ham's F10, Ham's F12, and RPMI 1640. In a preferred embodiment, the basal medium may further include at least one growth factor. Growth factors which may be employed include, but are not limited to, interleukins, such as interleukin-1 and interleukin-3; fibroblast growth factors; prolactin; growth hormone; transforming growth factors such as transforming growth factor-$\propto$; insulin-like growth factors, such as IGF-I and IGF-II; glucagon; insulin; platelet-derived growth factor; thyroid hormones, such as T3; hepatopoietins such as hepatopoietin A and hepatopoietin B; epidermal growth factors (EGF);

dexamethasone; norepinephrine; and transferrin. One or more of such growth factors may be contained in a serum-free medium referred to as hormonally-defined medium, or HDM. An example of HDM is further described in Enat, et al., Proc. Nat. Acad. Sci., Vol. 81, pgs. 1411-1415 (1984). Representative examples of hormonally defined medium (HDM), which may be prepared in RPMI 1640, Ham's F10, Ham's F12, or other basal media, include the following components in the following concentrations:

Component	HDM Concentration
Insulin	10μg/ml
Growth hormone	10μU/ml
Prolactin	20mU/ml
Glucagon	10μg/ml
EGF	50ng/ml
Dexamethasone	10^{-8}M
T3	10^{-9}M
Selenium	3×10^{-10}M
Copper	10^{-7}M
Zinc	10^{-10}M

The basal medium may further include a supplement such as, for example, bovine serum albumin, lipoproteins such as high density lipoproteins (HDL), and/or free fatty acids. Free fatty acids which may be contained in the supplement include, but are not limited to, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid, or mixtures thereof.

An example of a medium which contains such supplements contains Ham's F12 medium to which is added 0.4% bovine serum albumin, and 7.6mEq per liter of a free fatty acid mixture having the following free fatty acids in the following proportions:

Palmitic acid	31%
Palmitoleic acid	2.8%
Stearic acid	11.6%
Oleic acid	13.4%
Linoleic acid	35.6%
Linolenic acid	5.6%

Such medium is sometimes hereinafter referred to as medium HBF.

As a further representative example, either Ham's F12 medium or medium HBF may include the following growth factors, hormones, or other chemicals used as supplements in the concentrations given below:

Component	Concentration
Dexamethasone	10^{-6}M
Insulin	0.1 to 100μg/ml
Multi-Stimulating activity (MSA)*	50ng/ml
EGF	25 to 100ng/ml
Norepinephrine	10^{-4}M
Hepatopoietins	25μl/ml
FGF's	10ng/ml

*Obtained from Sigma Chemical Co., St. Louis, Mo. MSA contains insulin-like growth factor-I and insulin-like growth factor-II.

The number of such immature cells cultured under conditions such as those hereinabove described may be monitored by a variety of procedures. In general, the number of such cultured immature cells can increase by at least about 3- fold in a period of one week, preferably at least about 10- fold.

In one preferred embodiment, a human liver cell population, which has been enriched for hepatocyte precursors, is plated on or in a matrix of collagen Type IV under conditions in which

the cells could polarize and feed through a basal surface such as a Millicell support. The matrix-bound hepatocyte precursors would be provided with an embryonic liver-derived stromal cell feeder layer. The cells are cultured in a serum-free medium having less than 0.4mM calcium, and rich in free fatty acids. Some expansion of the hepatocyte precursors occurs under such conditions. If one desires to accelerate the expansion of the hepatocyte precursors, one may add growth factors to the medium. Extensive growth, or expansion may be obtained by adding cytokines such as interleukin-3 or interleukin-1 to the medium. The addition of growth factors such as, for example, epithelial growth factor (EGF), fibroblast growth factor (FGF), or IGF-II induces the expansion of a higher proportion of hepatocyte precursors.

Although Applicants have disclosed herein examples of preferred embodiments for the expansion of the immature cells, it is also contemplated that within the scope of the present invention, other methods may be employed for the expansion of such cells.

Applicants have found that by growing hepatocyte precursors in a medium which contains liver stromal cells and an extracellular matrix, one is able to support, or expand or proliferate the hepatocyte precursors. One may obtain growth en masse of the cells (i.e., diffuse, proliferative growth), or in some cases, the growth of colonies of such immature cells, also known as clonal growth, as well as enabling the cells to survive for extended periods of time.

Upon expansion of the hepatocyte precursors, such expanded hepatocyte precursors may be cultured under conditions which enable at least a portion of the hepatocyte precursors or at least a portion of the progeny of such hepatocyte precursors to differentiate into mature

hepatocytes; alternatively, such expanded hepatocyte precursors may be transplanted into a patient, preferably within the liver tissue, whereupon at least a portion of the hepatocyte precursors or a portion of the progeny of such hepatocyte precursors will differentiate into mature hepatocytes.

It is also contemplated that within the scope of the present invention, such hepatocyte precursors may be genetically engineered to express any of a wide variety of proteins or polypeptides.

Gene(s) of interest which may be expressed by the hepatocyte precursors, or the differentiated cells derived therefrom, include, but are not limited to: (1) gene(s) present in and expressed at biologically effective levels by normal liver cells, but present in and expressed in less than normal quantities in the liver cells of animals or human patients to be treated prior to transfer of gene(s) of interest into them; (2) gene(s) not expressed in normal mature liver cells; or (3) gene(s) expressed in normal mature liver cells but whose structure is defective in the animals or patients to be treated, leading to the production of a non-functional protein, alone or in any combination thereof.

The gene(s) of interest can be incorporated into the cellular genetic material (e.g., into genomic DNA) or can be present extrachromosomally (i.e., the gene persists as part of an episome and is expressed from the episome). The genetic material of interest can be DNA or RNA; the DNA can constitute all or a portion of a gene of interest (i.e., one whose expression in mature liver cells is desired).

The gene(s) incorporated into and expressed by the hepatocyte precursors or the differentiated cells derived therefrom can additionally include genetic material (e.g., DNA) encoding a selectable marker, which provides a means by which cells expressing the gene(s) of interest can be identified and selected. Hepatocyte precursors containing incorporated genetic material (i.e., gene(s) of interest and, optionally, genetic material encoding a selectable marker) are referred to as transduced hepatocyte precursors or genetically engineered hepatocyte precursors.

The gene(s) can be introduced, by means of an appropriate vector, into isolated and/or cultured hepatocyte precursors, which are subsequently transplanted into the recipient. Alternatively, a vector which recognizes a hepatocyte precursor as a target may be injected into the recipient, whereby the vector is incorporated into the hepatocyte precursors.

Such hepatocyte precursors to be genetically modified ex vivo can be obtained from a human or non-human animal, modified and returned to the same human or non-human animal by transplanting or grafting or, alternatively, can be obtained from a donor (i.e., a source other than the ultimate recipient), modified and placed into a recipient, again by transplanting or grafting.

The genetically engineered cells of the present invention may be employed in treating any disease which results from a single gene defect which can be corrected by expression of the normal gene in the hepatocyte precursors or the differentiated cells derived therefrom. Genetically engineered cells of the present invention may be used, for example, for the delivery of polypeptides or proteins which are useful in prevention and therapy of an acquired or an

inherited defect in hepatocyte (liver) function. For example, they can be used to correct an inherited deficiency of the low density lipoprotein (LDL) receptor, and/or to correct an inherited deficiency of ornithine transcarbamylase (OTC), which results in congenital hyperammonemia.

Such genetically engineered cells may also be employed in the treatment of hemophilia due to Factor VIII or Factor IX deficiency; alpha-1 anti-trypsin deficiency; phenylketonuria (PKU) or other illnesses resulting from defects in the urea cycle or other defects in metabolic pathways.

The genetically engineered hepatocyte precursors can be used to provide a desired therapeutic protein or peptide by a means essentially the same as that by which the protein or peptide is normally produced and, in the case of autologous grafting, with little risk of an immune response and graft rejection. In addition, there is no need for extensive (and often costly) purification of a polypeptide before it is administered to an individual, as is generally necessary with an isolated polypeptide. Such genetically engineered hepatocyte precursors produce the polypeptide as it would normally be produced.

Retroviral vectors may be used to transduce hepatocyte precursors with genetic material which includes gene(s) encoding polypeptide(s) or protein(s) of interest and/or genetic material encoding a dominant selectable marker.

Because genes can be introduced into hepatocyte precursors using a Retroviral vector, they can be under (subject to) the retroviral vector control; in such a case, the gene of interest is transcribed from a retroviral promoter. A promoter is a specific nucleotide sequence recognized

by RNA polymerase molecules that start RNA synthesis. Alternatively, retroviral vectors having additional promoter and regulatory elements (in addition to the promoter which is responsible for normal retroviral gene transcription), which are responsible for the transcription of the gene(s) of interest, can be used. This category includes, but is not limited to, promoters, enhancers, or other regulatory elements for genes normally expressed in the liver. For example, a construct in which there is an additional promoter modulated by an external factor or signal can be used, making it possible to control the level of polypeptide being produced by the modified hepatocyte precursors, or by mature hepatocytes which have differentiated from such precursors, by providing that external factor or signal. For example, heat shock proteins are proteins encoded by genes in which the promoter is regulated by temperature. In another example, the promoter of the gene which encodes the metal-containing protein metallothionine is responsive to cadmium (Cd) ions. Additional examples include promoters known to be responsive to cyclic AMP, or to glucocorticoids, or to interferons. Incorporation of these promoters or other promoters influenced by external signals also makes it possible to regulate the production of the polypeptide by the genetically engineered hepatocyte precursors or mature hepatocytes differentiated from such precursors.

It is also possible to use viruses other than retroviruses to genetically engineer or modify hepatocyte precursors. Gene(s) of interest can be introduced by means of any virus, or a vector derivative thereof which can express such gene(s) in such cells. For example, SV40, herpes virus, adenovirus, adeno-associated virus, Epstein-Barr virus, and papilloma virus can be used

for this purpose. DNA viruses or their vector derivatives can also be used to introduce gene(s) of interest, as well as a gene encoding a selectable marker, into such immature cells.

It is also contemplated that the hepatocyte precursors may be transduced with non-viral expression vehicles or DNA constructs, such as plasmids, for example.

Hepatocyte precursors expressing the gene(s) of interest may be grown in tissue culture vessels; removed from the culture vessel; and introduced into the body. This can be done surgically, for example. In this case, the tissue which is made up of transduced hepatocyte precursors capable of expressing the nucleotide sequence of interest is grafted or transplanted into the body. For example, it can be placed in the abdominal cavity in contact with/grafted onto the liver or in close proximity to the liver. Alternatively, the genetically engineered hepatocyte precursors can be attached to a support, such as, for example, microcarrier beads, which are introduced (e.g., by injection) into the peritoneal space of the recipient. Direct injection of genetically engineered hepatocyte precursors into the liver or other sites is also contemplated. Alternatively, the genetically engineered hepatocyte precursors may be injected into the portal venous system or may be injected intrasplenically. Subsequent to the injection of such cells into the spleen, the cells may be transported by the circulatory system to the liver. Once in the liver such cells may express the gene(s) of interest and/or differentiate into mature hepatocytes which express the gene(s) of interest.

Once introduced into the body of an individual, a portion of the genetically engineered hepatocyte precursors or a portion of their progeny differentiates into mature hepatocytes, which

provide a continuous supply of the protein, polypeptide, hormone, enzyme, or drug encoded by the gene(s) of interest. It is contemplated that such proteins, polypeptides or hormones may also be supplied by the hepatocyte precursors prior to, or in the absence of differentiation into mature hepatocytes. The amount of the protein, polypeptide, hormone, enzyme, or drug supplied in this way can be modified or regulated as needed.

Thus, the hepatocyte precursors are genetically engineered in such a manner that they produce a gene product (e.g., a polypeptide or a protein) of interest in biologically significant amounts. The hepatocyte precursors or the mature hepatocyte progeny therefrom, formed in this way can serve as a continuous drug delivery system to replace present regimens, which require periodic administration (by ingestion, injection, etc.) of the needed substance.

Genetically engineered hepatocyte precursors may be employed in the treatment of inherited disease and in the treatment of acquired disease. In the case of inherited diseases, this approach is used to provide genetically engineered hepatocyte precursors or mature hepatocytes differentiated therefrom, which contain DNA encoding a protein or polypeptide which an individual is unable to make correctly. Hepatocyte precursors of the present invention can also be used in the treatment of genetic diseases in which a product (e.g., LDL receptor) normally produced by the liver is not produced or is made in insufficient quantities. Here, hepatocyte precursors transduced with a DNA encoding the missing or inadequately produced substance can be used to produce it in sufficient quantities. In this case, at least a portion of the transduced hepatocyte precursors or a portion of their progeny differentiates into mature hepatocytes, which

would produce LDL receptors and thus provide a means of preventing or treating familial hypercholesterolemia. This is an inherited disease in which the primary genetic defect is an abnormality in the expression or function of the receptor for low density lipoproteins, leading to elevated levels of serum cholesterol and the premature development of coronary artery disease. The transduced hepatocyte precursors, and the mature hepatocytes differentiated therefrom could be used to produce sufficient quantities of the LDL receptor to overcome the underlying defect. This approach may also be extended to any patient having a predisposition to atherosclerosis due to hyperlipidemia.

There are also acquired diseases for which treatment can be provided through use of genetically engineered hepatocyte precursors. The genetically engineered hepatocyte precursors may also be employed to treat viral hepatitis, particularly hepatitis B or nonA-nonB hepatitis, by gene transfer. For example, a gene encoding an anti-sense gene could be introduced into hepatocyte precursors to inhibit viral replication. In this case, a vector including a structural hepatitis gene in the reverse or opposite orientation would be introduced into hepatocyte precursors, resulting in production in the genetically engineered hepatocyte precursors and any mature hepatocytes differentiated therefrom of an anti-sense gene capable of inactivating the hepatitis virus or its RNA transcripts. Alternatively, the hepatocyte precursors may be transduced with a gene which encodes a protein, such as, for example, ∝-interferon, which may confer resistance to the hepatitis virus.

Advantages of employing hepatocyte precursors of the present invention include the provision of a model system for the growth of hepatocyte precursors and/or the differentiation of such hepatocyte precursors into mature hepatocytes. Such a model system of hepatocyte precursors has greater growth potential than cultures of mature hepatocytes, and thus is better suited for various studies of liver cells, such as toxicology studies, carcinogenic studies, and vaccine production. Also, because such hepatocyte precursors may be dissociated from liver tissue and then be enriched and expanded, such expanded hepatocyte precursors obtained from one liver may thus be administered therapeutically to a plurality of patients. The administration of such immature cells may also be less likely to stimulate immune rejection than the injection of mature hepatocytes. In addition, mature hepatocytes may have a limited life span and may undergo a limited number of cell divisions, whereas hepatocyte precursors have a greater capacity to generate daughter cells. Thus, the life span of such a system may be significantly prolonged and possibly may be indefinite.

Examples of non-therapeutic uses of hepatocyte precursors include research of liver embryology, liver cell lineages, and differentiation pathways; gene expression studies; mechanisms involved in liver injury and repair; research of inflammatory and infectious diseases of the liver; studies of pathogenetic mechanisms; and studies of mechanisms of liver cell transformation and etiology of liver cancer. Additional therapeutic uses include liver transplantation for patients with liver failure due to alcoholism, infection, congenital liver diseases, etc., gene therapy for liver diseases that are genetically based such as, for example,

Wilson's disease, glycogen storage diseases, urea cycle enzyme defects, and Creigler-Najir disease; and the use of such hepatocyte precursors and any lineages of adult cells derived from them in assays for chemotherapy (eg., for liver cancers), for the production of vaccines for viruses that grow in the liver, and for studies of alcoholic cirrhosis. The hepatocyte precursors cells may also be employed as part of an "artificial liver;" i.e., the hepatocyte precursors may be placed in a container or apparatus, in which the hepatocyte precursors generate a liver lineage and function as a liver outside of the body. The container or apparatus is connected to the circulatory system of a human or animal subject.

In accordance with another aspect of the present invention, there is provided a composition comprising an animal cell population derived from liver. The cell population contains immature cells which are characterized by expression of alpha-fetoprotein or lack of essential expression of alpha-fetoprotein and albumin, and at least a portion of such cells or of the progeny of such cells is capable of differentiating into adult liver cells. The cells have been cultured under conditions which result in expansion of the immature cells. Such immature cells may be obtained from the livers of human or non-human animals hereinabove described. Although the progeny of such immature cells may differentiate into hepatocytes, such immature cells may differentiate into adult liver cells other than hepatocytes, such as bile duct cells, liver endothelial cells, and lipid-containing liver cells known as Ito cells.

Such immature cells derived from liver may be obtained from liver tissue and enriched or expanded under conditions hereinabove described for the enrichment and expansion of the

above-described hepatocyte precursors. It is also contemplated that such immature cells may be genetically engineered through techniques such as those hereinabove described, whereby such genetically engineered cells may be administered to an animal or a human subject, in which the genetically engineered cells and/or their differentiated progeny express gene(s) of interest.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

What is claimed is:

1. A composition comprising a cell culture of immature animal cells, including liver, pancreas, gut, lung, or bone marrow cells, which contains at least a population of hepatocyte precursor cells capable of differentiating into hepatocytes.

2. The composition of claim 1, wherein the hepatocyte precursor cells are capable of differentiating into hepatocytes in a serum-free culture medium comprising extracellular matrix and liver stromal cells.

3. The composition of claim 2, wherein the extracellular matrix is formed from a material comprising collagen, fibronectin, laminin or combinations thereof.

4. The composition of claim 3, wherein the collagen is type IV collagen.

5. The composition of claim 3, wherein the collagen is used alone or in combination with proteoglycans, or tissue extracts enriched in extracellular matrix materials.

6. The composition of claim 2, wherein the extra cellular matrix is coated upon a porous solid support.

7. The composition of claim 6, wherein the solid support comprises Millicell membrane support, filters, sponges, and hollow fiber systems.

8. The composition of claim 2, wherein the liver stromal cells are embryonic liver stromal cells.

9. The composition of claim 2, wherein the liver stromal cells are fetal liver stromal cells.

10. The composition of claim 1 which comprises a growth factor.

11. Genetically engineered hepatocyte precursor cells obtained by genetically engineering expanded hepatocytes precursor cells derived from culturing immature animal cells that contain at least a population of hepatocyte precursor cells capable of differentiating into hepatocytes.

12. The genetically engineered hepatocyte precursor cells of claim 11, wherein the hepatocyte precursor cells are differentiated into hepatocytes in a serum-free culture medium comprising extracellular matrix and liver stromal cells.

13. The genetically engineered hepatocyte precursor cells of claim 11, wherein the immature animal cells are selected from the group consisting of liver, pancreas, gut, lung, or bone marrow cells.

14. Genetically engineered hepatocyte precursor cells obtained by culturing immature animal cells including liver, pancreas, gut, lung, or bone marrow cells, that contain at least a population of hepatocyte precursor cells capable of differentiating into hepatocytes in a serum-free culture medium, that comprises extracellular matrix and liver stromal cells to provide expanded hepatocyte precursor cells and genetically engineering the expanded hepatocyte precursor cells.

15. The genetically engineered hepatocyte precursor cells of claim 14, wherein the liver stromal cells are embryonic liver stromal cells or fetal liver stromal cells.

16. The genetically engineered hepatocyte precursor cells of claim 11, wherein the genetic engineering comprises *ex vivo* genetic modification of the hepatocyte precursors.

17. The genetically engineered hepatocyte precursor cells of claim 16, wherein *ex vivo* genetic modification comprises obtaining hepatocyte precursor cells from a human or non-human subject, genetically modifying the hepatocyte precursor and transferring the genetically modified hepatocyte precursor cells to the same or a different human or non-human subject.

18. The genetically engineered hepatocyte precursor cells of claim 17, wherein said transferring comprises transplanting or grafting.

19. The genetically engineered hepatocyte precursor cells of claim 11, wherein genetically engineering comprises transducing hepatocyte precursor cells with a retroviral vector comprising a genetic material that encodes polypeptides or protein of interest and/or a dominant selectable marker.

20. The genetically engineered hepatocyte precursor cells of claim 11, wherein the genetic material is under the control of retroviral vector regulatory elements and/or regulatory elements of genes normally expressed in the liver.

ABSTRACT OF THE DISCLOSURE

A composition which comprises an animal cell population which contains immature animal cells. The immature animal cells are characterized by expression of alpha-fetoprotein or lack of essential expression of alpha-fetoprotein and albumin, and at least a portion of said immature animal cells or at least a portion of the progeny of said immature animal cells is capable of differentiating into cells which express albumin. The cell population is cultured under conditions which result in expansion of the cells. Expansion of the cells may be achieved by culturing the cells in the presence of an extracellular matrix and liver stromal cells; and preferably in the presence of growth factors. Such cells may be used for liver transplantation, artificial livers, and for toxicology and pharmacology studies. Such cells may also be genetically engineered to express proteins or polypepetides of interest.

Docket No.: 112787-110 PATE

DECLARATION AND POWER OF ATTORNEY

As a below named inventors, we hereby declare that:

My residence, post office and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names listed below) of the subject matter claimed and for which a patent is sought on the invention entitled PROLIFERATION HEPATOCYTE PRECURSORS, the specification of which
[ ] is attached hereto OR [X] was filed on November 18, 1996 as Application Serial No. 08/751,546

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by amendment referred to above.

I acknowledge the duty to disclose information which is known to me to be material to patentability in accordance with Title 37, Code Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application for patent or inventor's certificate, or Section 365(a) of any PCT international application which designated at least one country other t the United States, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing d before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed	
Number	Country	Day/Month/Year filed	Yes	No

I hereby claim the benefit under 35 USC §119(e) of any United States provisional application(s) listed below.

Prior Provisional Application(s):

Application Number	Filing Date

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or Section 365(c) of any P international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this applicat is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, Uni States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Sect 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application

Prior U. S. Application(s):

Serial No.	Filing Date	Status: Patented, Pending, Abandoned
07/741,128	8/7/91	Abandoned
08/265,696	6/24/94	Patented

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false stateme may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby grant(s) the firm of PEPPER HAMILTON LLP the power to insert on this Declaration any further identification including the application number and filing date, which may be necessary or desirable in order to comply with the rules of the United State Patent and Trademark Office for recordation of this document

I hereby appoint the following attorney(s) and/or agent(s) listed at the follwing customer number:

021289

PATENT AND TRADEMARK OFFICE

with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and all future correspondence should be addressed to the address at the aforementioned customer number, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and all future correspondence should be addressed to them.

Full name of sole or first Inventor: Lela M. Reid

Inventor's signature: Lela M Reid Date: May 21, 1998

Residence: Chapel Hill, North Carolina

Citizenship: USA

Post Office Address: 3621 Sweeten Creek Road, Chapel Hill, North Carolina 27514 USA

Full name of second joint inventor: Maria Agelli

Inventor's signature: Date:

Residence: New Providence, New Jersey

Citizenship: USA

Post Office Address: 77 Pearl Street, New Providence, New Jersey

Full name of second joint inventor: Andreas Ochs

Inventor's signature: Andreas Ochs Date: May 24, 1998

Residence: Bronx, New York

Citizenship: German

Post Office Address: 1579 Rhinelander Avenue, Apt. 2-J Bronx, New York
(in Germany): Habichtweg 8, Frieburg 791101 GERMANY

Freiburg

Docket No.: 112787-110 PATEN

DECLARATION AND POWER OF ATTORNEY

As a below named inventors, we hereby declare that:

My residence, post office and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names listed below) of the subject matter claimed and for which a patent is sought on the invention entitled PROLIFERATION HEPATOCYTE PRECURSORS, the specification of which
[ ] is attached hereto OR [X] was filed on November 18, 1996 as Application Serial No. 08/751,546

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by amendment referred to above.

I acknowledge the duty to disclose information which is known to me to be material to patentability in accordance with Title 37, Code Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application for patent or inventor's certificate, or Section 365(a) of any PCT international application which designated at least one country other t the United States, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing d before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed	
Number	Country	Day/Month/Year filed	Yes	No

I hereby claim the benefit under 35 USC §119(e) of any United States provisional application(s) listed below.

Prior Provisional Application(s):	
Application Number	Filing Date

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or Section 365(c) of any I international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this applica is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, Un States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Sec 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this applicatio

Prior U. S. Application(s):		
Serial No.	Filing Date	Status: Patented, Pending, Abandoned
07/741,128	8/7/91	Abandoned
08/265,696	6/24/94	Patented

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belie believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so mad punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false staten may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby grant(s) the firm of PEPPER HAMILTON LLP the power to insert on this Declaration any further identification, including the application number and filing date, which may be necessary or desirable in order to comply with the rules of the United States Patent and Trademark Office for recordation of this document.

I hereby appoint the following attorney(s) and/or agent(s) listed at the following customer number:

021269

PATENT AND TRADEMARK OFFICE

with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and all future correspondence should be addressed to the address at the aforementioned customer number, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and all future correspondence should be addressed to them.

Full name of sole or first inventor: Lola M. Reid

Inventor's signature: Lola M. Reid Date: May 21, 1998

Residence: Chapel Hill, North Carolina

Citizenship: USA

Post Office Address: 3621 Sweeten Creek Road, Chapel Hill, North Carolina 27514 USA

Full name of second joint inventor: Maria Agelli

Inventor's signature: Maria Agelli Date: May 26, 199[illegible]

Residence: New Providence, New Jersey

Citizenship: USA

Post Office Address: 77 Pearl Street, New Providence, New Jersey

Full name of second joint inventor: Andreas Ochs

Inventor's signature: Date:

Residence: Bronx, New York

Citizenship: German

Post Office Address: 1579 Rhinelander Avenue, Apt. 2-J Bronx, New York
(in Germany): Habichtweg 6, Frieburg 791101 GERMANY